NITRATION OF FUNCTIONAL TYROSYL RESIDUES IN RABBIT MUSCLE PHOSPHORYLASE b

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SUMMARY

Upon reaction of rabbit muscle phosphorylase b with tetranitromethane in a stoichiometric ratio with respect to the tyrosyl content, 2 out of 34 phenolic groups per mole of monomer (M.W. 95,000) were nitrated with an almost complete loss of activity. Only one residue per monomer was nitrated in the presence of AMP, the major part of the activity being preserved. The sedimentation pattern of modified phosphorylase b showed that, following nitration in the absence of AMP, the enzyme was fully dissociated into monomers, whereas, when the enzyme was nitrated in its presence, the dimeric structure was retained.

INTRODUCTION

The catalytic function of rabbit muscle phosphorylase b (EC 2.4.1.1) was found to be strictly dependent upon some tyrosyl residues. The allosteric activator, AMP, selectively protected two of these residues, essential for activity, towards reversible chemical modification by N-acetylimidazole (1). In an effort to further clarify the role of phenolic groups in the enzyme, we have investigated the effect of the reaction with tetranitromethane (TNM[1]), a highly specific reagent for tyrosyl residues (2).

Phosphorylase b was allowed to react with TNM in a stoichiometric ratio with respect to tyrosyl residues; under these conditions, only 2 out of 34 residues per mole of monomer were modified, with an almost complete loss of enzymic activity. The presence of AMP protected one tyrosyl residue and drastically reduced enzyme inactivation. Sedimentation analysis proved that these effects were paralleled by profound modifications in the association-dissociation equilibrium of the protomers.

EXPERIMENTAL

Materials. Rabbit muscle phosphorylase b was prepared according to Fisher and Krebs (3) except that 0.5 mM dithiothreitol was substituted for cysteine in the crystallization steps. AMP-free enzyme was obtained by gel filtration on a Sephadex G-25 column (1.5 x 25 cm), equilibrated in 25 mM β-glycerophosphate, pH 7.0 and 0.5 mM dithiothreitol. TNM was from Pierce, 5'-AMP, $NADP^+$, phosphoglucomutase and glucose-6-phosphate dehydrogenase were from Boehringer;

[1]Abbreviation used: TNM, tetranitromethane.

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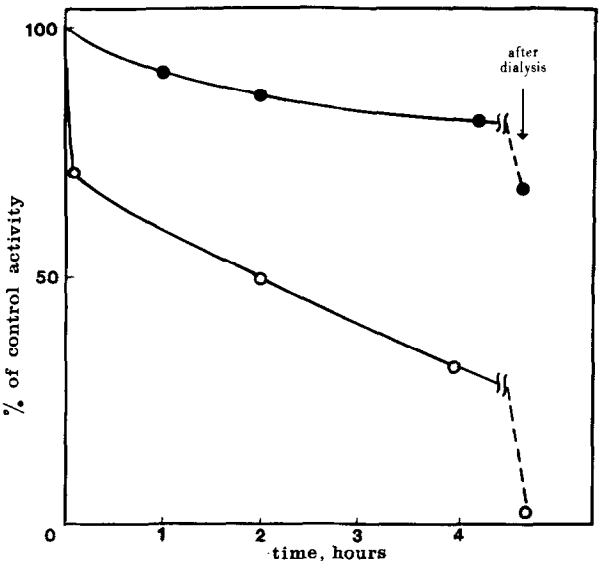

Fig. 1 - Reaction of phosphorylase b with TNM at 0°C. 1 mg enzyme was incubated as described under Experimental in the absence (○) and presence (●) of AMP, 2 mM final concentration. TNM/tyr molar ratio was 1.

AMP-free glycogen was from Sigma. All other chemicals were of the highest purity commercially available.

Enzyme assay. Activity was measured at 20°C in the direction of glycogen breakdown according to Helmreich and Cori (4). NADPH formation was recorded at 340 nm in a Gilford Model 2400S spectrophotometer; a molar extinction coefficient of 6.22×10^3 was used (5). The specific activity of the crystalline enzyme was such that 1 mg released 55 µmol of inorganic phosphate per min. Protein was determined according to Lowry et al. (6).

TNM reaction. Nitration of the protein (2 mg/ml) was performed at 0°C in 25 mM β-glycerophosphate, pH 7.0, 5 mM thiodiglycol, by adding the appropriate amount of a 0.5 M ethanolic solution of TNM, as described by Riordan et al. (2). Aliquots were withdrawn at different times and assayed for enzymic activity. Nitrated phosphorylase b was dialyzed against 25 mM β-glycerophosphate, pH 7.0. The extent of tyrosyl nitration was measured either colorimetrically (7) or by quantitation of 3-nitrotyrosine using a JEOL Model JLC-6AH aminoacid analyzer.

Sedimentation. Sedimentation experiments on dialyzed samples were carried out in a Spinco Model E analytical ultracentrifuge at 20°C, 59,780 rpm; photographs were taken at intervals of 4 min after reaching full speed.

RESULTS

Reaction of phosphorylase b with TNM at 0°C, at a TNM/tyr molar ratio of 1, produced a time-dependent inactivation of the enzyme. The extent of the inactivation was over 95%, following dialysis of the excess of reagent (Fig. 1). In the presence of the allosteric activator, AMP, the nitrated enzyme retained 70% of the original activity. Colorimetric determination of 3-nitrotyrosyl residues showed that enzyme inactivation, in the absence of

TABLE 1

Reactivity of tyrosyl residues of phosphorylase b (34 Tyr/95,000) with TNM.

	TNM/tyr = 9		TNM/tyr = 5		TNM/tyr = 1	
	No. of modified Tyr	% control activity	No. of modified Tyr	% control activity	No. of modified Tyr	% control activity
Unprotected enzyme	10.2	2	4.0	2	2.1	3
AMP-protected enzyme	10.1	2	2.2	9	1.0	69

AMP, occurred in parallel with nitration of two residues per monomer (M.W. = 95,000). The protection observed by AMP reduced the extent of modification to only one residue per monomer (Table 1). Aminoacid analysis confirmed the colorimetric data. The Table also shows the stoichiometry of 3-nitrotyrosyl formation at two higher TNM/tyr molar ratios. In particular, at a TNM/tyr ratio of 9, ten tyrosyl residues were modified, in both the presence and absence of AMP, and almost complete inactivation was observed in either case.

Sedimentation experiments were performed on the modified protein in order to establish a correlation between the state of aggregation of enzyme protomers and the extent of chemical modification. Fig. 2 shows the sedimentation pattern of native and nitrated phosphorylase b. The native enzyme exhibited a sedimentation behaviour consistent, in the conditions of the run, with the existence of a dimer-tetramer equilibrium (8); unprotected phosphorylase b, nitrated at a TNM/tyr molar ratio of 1, was almost completely dissociated into monomers, whereas the sedimentation pattern of the AMP-protected enzyme showed that the presence of the effector largely prevented such dissociation; in both cases the fast sedimenting peak corresponding to the tetramer was no longer observed. When the reaction was carried out at a 9-fold molar excess of TNM, the enzyme was completely dissociated into monomers, regardless of the presence of AMP during nitration.

DISCUSSION

The finding that a limited number of tyrosyl residues were involved in the catalytic and allosteric features of rabbit muscle phosphorylase b (1) prompted an investigation on the effect of a reagent, TNM, which specifically nitrates tyrosyl residues at position 3 of the benzene ring. Conditions

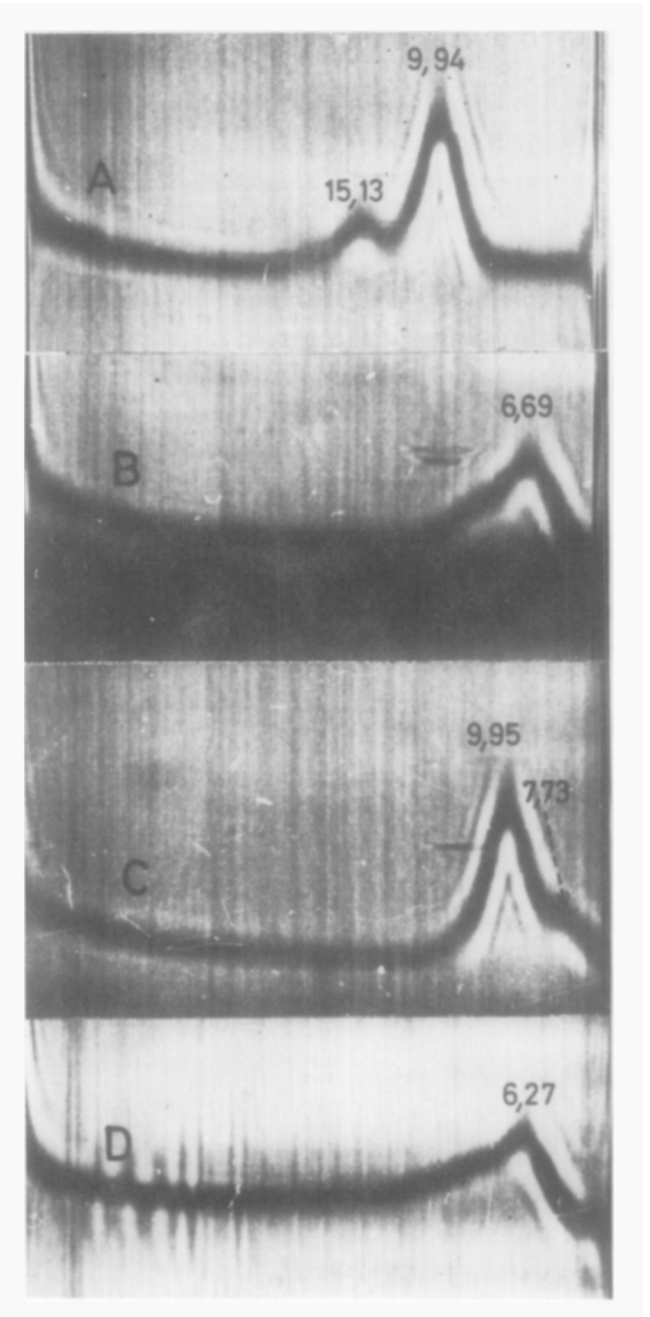

Fig. 2 - Sedimentation pattern of phosphorylase b nitrated in the presence and absence of AMP. Photographs were taken 12 min after reaching top speed. Sedimentation was from right to left. Enzyme concentration, approx. 2 mg/ml. A, native; B, nitrated, unprotected; C and D, nitrated, AMP-protected. The TNM/tyr molar ratio was 1 in B and C; 9 in D; $S_{20°C, w}$ values are indicated in the Figure.

were found under which the changes in enzymic activity which accompanied nitration of both the unprotected and AMP-protected enzyme, were similar to those found upon reaction with N-acetylimidazole; on the other hand, the number of chemically modified tyrosyl residues was significantly reduced, namely two in the unprotected and one in the AMP-protected enzyme. Sulfhydryl determination (9) indicated that, under the chosen conditions, no cysteyl residues were oxidized by TNM.

The data presented here show that nitration of these two residues is accompanied by a drastic change in the quaternary structure, the monomer becoming the dominant species. The protection afforded by AMP, which resulted in the reaction of only one tyrosyl residue per monomer, with retention of the major part of the activity, had its structural counterpart in that the dimer, the catalytically active species, was preserved. Thus, it appears that one specific tyrosyl residue of phosphorylase b is essential to enzymatic activity; nitration of this residue probably triggers a conformational rearrangement of the protein with a consequent change in the association equilibrium of protomers.

It should be stressed that the tetrameric form is totally absent (Fig. 2C) in the mononitrated (AMP-protected) enzyme; this observation, together with the small but significant decrease in activity occurring in mononitrated phosphylase b, indicates that also the phenolic group modified in the presence of the protecting ligand may play a role in the maintenance of the catalytic and structural features of the enzyme, although its modification does not lead to dissociation into monomers. Studies will therefore be devoted to a further characterization of the mononitrated protein.

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